IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 10/677,733	Customer No.: 23379
Applicant: Gardner et al.	Confirmation No. 4887
Filed: Oct 01, 2003	Group Art Unit: 1656
Docket No. UTSD:1510	Examiner: Noakes, Suzanne Marie

Title: NMR Detection of Foreign PAS Domain Ligands

BRIEF ON APPEAL

The Honorable Board of Appeals and Interferences
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Honorable Board:

We appeal from the Examiner's Aug 06, 2008 final rejection of claims 3 and 5.

REAL PARTY IN INTEREST

The real party in interest is the Board of Regents, the University of Texas System, the assignee of this application.

RELATED APPEALS AND INTERFERENCES

A prior appeal in this application was decided by the Board on Sep 19, 2007. Appellants are unaware of any other related appeals or interferences.

STATUS OF CLAIMS

Claims 3 and 5 are rejected and subject to this appeal; claim 4 is objected to as being dependent on a rejected claim; claims 1, 2 and 6 are canceled.

STATUS OF AMENDMENTS

All Amendments are believed to be properly before the Board.

SUMMARY OF CLAIMED SUBJECT MATTER

A method of detecting binding of a PAS (Per-ARNT-Sim) domain of a protein with a foreign core ligand of the PAS domain, wherein the PAS domain is prefolded in its native state, the method comprising the steps of: (a) determining from NMR analysis of the PAS domain that the PAS domain comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity; (b) detecting a first NMR spectrum of the PAS domain in the presence of a the foreign ligand; (c) comparing the first NMR spectrum with a second NMR spectrum of the PAS domain in the absence of the ligand; and therefrom (d) determining the presence of the ligand specifically bound within the hydrophobic core of the PAS domain. Specification, p.2, line 25 – p.3, line 1; claim 3.

In a particular embodiment, the protein consists of the PAS domain. Specification, p.5, line 22; claim 5.

GROUND OF REJECTION TO BE REVIEWED ON APPEAL

I. DOES THE ACTION ESTABLISH THAT CLAIMS 3 AND 5 ARE UNPATENTABLE OVER FESIK (WO97/18471) IN VIEW OF ANY OF US PATENTS 5,843,683 (EDERY ET AL.); 6,291,429 (TAKAHASKI ET AL.); 6,436,654 (BERKENSTAM ET AL.) UNDER 35USC103(a)?

ARGUMENT

I. THE ACTION DOES NOT ESTABLISH THAT CLAIMS 3 AND 5 ARE UNPATENTABLE OVER FESIK (WO97/18471) IN VIEW OF ANY OF US PATENTS 5,843,683 (EDERY ET AL.); 6,291,429 (TAKAHASKI ET AL.); 6,436,654 (BERKENSTAM ET AL.) UNDER 35USC103(a).

Relevant background of the invention is provided in the Specification at p.1, line 17 – p.2, line 5:

> PAS (Per-ARNT-Sim) domains are protein interaction domains widely used for intra- and intermolecular associations. Database searches indicate that the PAS domain family contains over 3000 members distributed in all kingdoms of

life. Structural studies reveal a common mixed α/β fold predicted to be present in all members of this family (Crews & Fan, 1999; Pellequer et al., 1998).

Some members of the PAS family are known to contain small molecules within their cores, allowing them to sense stimuli and regulate diverse biological processes. For example, heme binding by the PAS domains of FixL (Gong et al., 1998; Miyatake et al., 2000) and Dos (Delgado-Nixon et al., 2000) allows bacteria to sense oxygen levels; blue light photoreception in plant phototropins is achieved through a flavin molecule associated with their LOV domains (a PAS domain subclass) (Crosson et al., 2003); and binding of exogenous organic compounds by the C-terminal PAS domain of the aryl hydrocarbon receptor (AhR) displaces a chaperone protein, induces a conformational change and activates the transcription of xenobiotic metabolizing enzymes (Schmidt & Bradfield, 1996). In all these examples, the cofactor is reportedly required for proper folding and functioning of the PAS domain within the context of the holo-protein.

However, for most PAS domains there is no evidence for such a cofactor. In fact, structurally characterized PAS domains without bound cofactors (Amezcua et al., 2002; Erbel et al., 2003; Morais Cabral et al., 1998) show tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site.

Fesik (WO97/18471) discloses the use of particular two-dimensional $^{15}N/^{1}H$ NMR correlation spectra to identify ligand binding to target biomolecules. Fesik teaches nothing about PAS domains.

Edery (US 5,843,683) characterizes four PAS domain containing proteins (AHR, SIM, ARNT and PER) and use co-immunoprecipitation experiments to propose that PAS domains engage in PAS-PAS interactions. Edery proposes and claims assays for molecules that modulate PAS-PAS interactions.

Takahaski (US 6,291,429) describes circadian clock genes from humans and mice, and proposes contemplated uses of CLOCK polypeptides including use "in a screening assay for the

identification of drugs or compounds that inhibit the action of CLOCK polypeptide (e.g., DNA binding)." Takahaski, col.9, lines 13-27.

Berkenstam (US 6,436,654) discloses and claims methods for identifying compounds which modulate the function of a functional domain of a variant of human HIF-1α that lacks at least one functional domain thereof.

Prior to our invention it was known to use NMR to identify ligand binding to target molecules. Prior to our invention it was known that PAS domains are protein interaction domains widely used for intra- and intermolecular associations. Prior to our invention it was known that there were two structurally and functionally distinct classes of PAS domains: one kind (e.g. PYP PAS) purified with a core-bound cofactor required for proper folding and formation, and crystallographic analysis showed the cofactor bound inside a core pocket; the other class (e.g. HERG PAS) did not purify with a core-bound ligand, and crystallographic analysis revealed a tight core with no apparent core binding pocket. It is the latter class which is the subject of our claims, and we whole-heartedly agree with the Board's conclusion that the prior art suggests utilizing this class of PAS domains (without bound cofactors and showing tightly packed cores with no pre-formed cavities) for ligand screening:

> Takahaski suggests a method for identifying ligands for a PAS protein having a hydrophobic core (Answer 6). Edery also describes an assay method for identifying compounds that regulate a PAS domain protein's activity. Thus, despite the fact that these proteins have tightly packed cores with no pre-formed cavities - a fact that Appellants have not challenged - it was still suggested that these PAS domain proteins be utilized for ligand screening (see, e.g., Takahaski, at col. 9, ll. 14-16; Edery, at col. 46-50).
>
> Decision, para. bridging p.6 and 7.

Prior to our invention the N-terminal PAS domain of the HERG potassium channel was postulated to self-regulate the HERG protein by binding the channel body, and we similarly whole-heartedly concur with the prior Board Decision's conclusion that it would have been

obvious to try to target this activity with regulatory small molecules:

> The Specification refers to various prior art publications, including Morais Cabral *(Cell,* 95:649-655, 1998), for teaching "structurally characterized PAS domains without bound cofactors (... Morais Cabral et al., 1998) showing tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site" (Spec. 2: 2-5). Morais Cabral, which Appellant admits satisfies the claim limitations for a PAS domain (App. Br. 5), compares the eag PAS domain of the HERG potassium channel to other PAS domain proteins known to comprise a ligand in their hydrophobic core *(see id.,* at 852, col. 2, describing the PYP photoreceptor which has a chromophore associated with its PAS domain). Morais Cabral conclude: "[g]iven the regulatory roles of PAS domains in other protein systems, we suspect that the eag domain will have a dynamic influence on the gating of the HERG K+ channels through the binding of small molecule or protein effectors" *(id.,* at 854, col. 2). Thus, despite having a tightly packed core with no pre-formed cavity, in view of its similarity to other PAS domain proteins, Morais Cabral suggested that small molecules might regulate eag domain activity as they do for other PAS domains.
>
> Decision, para. bridging p.7 and 8.

Our dispute lies in exactly what kind of screening assay the prior art suggests. We contend that the screening assays suggested by the prior art are not what we are claiming.

For example, Morais Cabral used scanning mutagenesis to identify a hydrophobic patch on the surface of the HERG PAS domain that forms an interface with the body of the potassium channel to which it tightly binds. To do this, they introduced various point mutations <u>on the surface</u> of the PAS domain and then assessed their functional impact by monitoring channel deactivation in electrophysiological recordings of Xenopus oocytes expressing the mutant proteins. Id., Fig. 5B-C. Morais Cabral found that the presence of the PAS domain bound to the channel via that surface patch slows the rate of channel deactivation, suggesting a regulatory function. Id., Abstract; p.649, col.2, first full para.; p.652, Fig. 5A and first and second full

paragraphs. Morais Cabral's regulation can visualized by imagining the red PAS domain of Fig. 1A swinging up and binding (via its hydrophobic surface patch – green in Fig. 5A) to the blue channel core. What Morais Cabral is suggesting is that small molecules or effector proteins might target and interfere with or regulate this intramolecular PAS-channel binding. To the extent this suggests a screening assay to identify foreign small molecule regulators, Morais Cabral provides a functional electrophysiological assay for doing just that. Instead of screening regulatory point mutations, the same methods could be used to screen for regulatory small molecules.

The cited Edery (US 5,843,683) and Takahaski (US 6,291,429) similarly propose screening compounds in functional assays. In particular, Edery proposes in vivo, transcriptional reporter assays for PAS dimerization:

> In the third aspect, the invention features an in vivo assay method for identifying, screening and characterizing compounds potentially useful for treatment of diseases or disorders arising from abnormal PAS-PAS binding affinities. The method includes transfecting purified nucleic acid encoding a PAS-containing protein into a host cell which contains, or is manipulated to contain nucleic acid of a reporter gene whose transcription is regulated by the presence or absence of dimerized PAS-containing proteins. Test sample compounds can then be introduced and the effect on reporter gene transcription assayed.
> Edery, col.3, lines 46-50.

The cited Berkenstam (US 6,436,654) similarly discloses methods for identifying compounds which modulate the "function" of HIF-1α, and the only specifically disclosed assay is a transcriptional reporter read-out assay. Berkenstam, Figs. 4-13; Example 5 at col. 13-14.

And the cited Takahaski proposes similarly inhibiting functional activity of the CLOCK polypeptide, such as DNA binding:

> A CLOCK polypeptide of the present invention has numerous uses. By way of example, such a polypeptide can be used in a screening assay for the identification of drugs or compounds that inhibit the action of CLOCK

> polypeptide (e.g., DNA binding). The CLOCK polypeptide is an integral component of the circadian clock of mammals. As set forth below, animals lacking the ability to produce the CLOCK polypeptide have significant dysfunctions in their circadian clock. Mutant animals producing an altered CLOCK polypeptide can be given the normal CLOCK polypeptide together with suspected agonists or antagonists and the effects of such treatment on the restoration of a normal circadian rhythm can be determined. The CLOCK polypeptide can also be used to treat animals having circadian rhythm dysfunctions as set forth hereinafter. Takahaski, col.9, lines 13-27.

No where do any of these references suggest screening for small molecules that simply bind the subject PAS domains. A small molecule that simply bound the subject PAS domain would be of no interest to the authors of the cited art. Morais Cabral expresses no interest in identifying a small molecule that simply binds the HERG PAS domain; rather, all that Cabral is interested in, and all that his assay will even detect, are compounds which interfere with channel activation as measured in his electrophysiological assays. Hence, we can not agree that these references suggest screening for compounds in an in vitro binding assay format, such as NMR.

In any event, even if such as suggestion is inferred, it does not reach our claims. For example, to the extent Morais Cabral was interested in PAS binding compounds, he would be interested in compounds which bound the hydrophobic surface patch interface and thereby had the potential to interfere with its function. No where does Morais Cabral or any of these references suggest targeting the hydrophobic core of the subject PAS domains. The crystal structures of these PAS domains show a tight core with no apparent binding cavity. The 15 point mutations Morais Cabral analyses were chosen to be "spread over the domain surface" (p.651, first full para; Fig. 5A) – not within the interior of the domain, which Morais Cabral had no reason to target. None of Morais Cabral, Takahaski, Berkenstam, or Edery had or offers any reason to suspect the tightly-packed core of their respective PAS domain could bind small molecules, or if it could, that such binding would induce surface changes that would in turn impact the domain's function in a manner relevant to their functional assays.

Our claims are specifically directed to a method of detecting binding of a PAS domain of a protein with a foreign core ligand of the PAS domain, wherein the PAS domain is prefolded in its native state. The method specifically requires the steps of: (a) determining from NMR analysis of the PAS domain that the PAS domain comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity; (b) detecting a first NMR spectrum of the PAS domain in the presence of a foreign ligand; (c) comparing the first NMR spectrum with a second NMR spectrum of the PAS domain in the absence of the ligand; and (d) determining the presence of the ligand specifically bound within the hydrophobic core of the PAS domain. The cited art does not suggest applying this particular screening assay to PAS domains with a hydrophobic core having no NMR-apparent a priori formed ligand cavity. There is no motivation in the cited art to specifically assay core ligand binding as claimed.

In a particular embodiment, the protein consists of the PAS domain. Specification, p.5, line 22; claim 5. Here, there is no other domain to participate in any intra- or inter-molecular interaction, further distinguishing cited art which rely on functional interaction with non-PAS domains.

Though the cited art does not support a prima facie case for obviousness, for good measure we provided affirmative evidence documenting the fact that one skilled in the art would have considered the claimed invention nonobvious at the time it was made (see, Declarations of Professors Stephen R. Sprang and Kevin H. Gardner).

As the cited art does not establish that claims 3 and 5 are unpatentable, Appellants respectfully request reversal.

Please charge our Deposit Account No.19 0750 (order UTSD:1510) all necessary fees for this communication.

Respectfully submitted,
SCIENCE & TECHNOLOGY LAW GROUP

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CLAIMS APPENDIX

1-2 (canceled)

3. A method of detecting binding of a PAS (Per-ARNT-Sim) domain of a protein with a foreign core ligand of the PAS domain, wherein the PAS domain is prefolded in its native state, the method comprising the steps of:

determining from NMR analysis of the PAS domain that the PAS domain comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity;

detecting a first NMR spectrum of the PAS domain in the presence of the foreign ligand;

comparing the first NMR spectrum with a second NMR spectrum of the PAS domain in the absence of the ligand; and therefrom

determining the presence of the ligand specifically bound within the hydrophobic core of the PAS domain.

4. (objected) The method of claim 3, wherein the PAS domain is the PAS A domain of PAS kinase.

5. The method of claim 3 wherein the protein consists of the PAS domain.

6. (canceled)

EVIDENCE APPENDIX

Declaration under 37CFR1.132 by Professor Stephen R. Sprang dated Nov 19, 2007, entered by Final Action dated 12/11/2007

Declaration under 37CFR1.132 by Professor Kevin H. Gardner dated Apr 10, 2008), entered by Final Action dated 08/06/2008

Morais Cabral (Cell, 95:649-655, 1998) cited in prior Board Decision in Appeal 2007-2956 in this application, decided Sep 19, 2007.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 10/677,733	Customer No.: 23379
Applicant: Gardner et al.	Confirmation No. 4887
Filed: Oct 01, 2003	Group Art Unit: 1656
Docket No. UTSD:1510	Examiner: Nashed, Nashaat T.

Title: NMR Detection of Foreign PAS Domain Ligands

DECLARATION UNDER 37CFR1.132

I, Professor Stephen R. Sprang, declare and state as follows:

1. I am a Professor in the Division of Biological Sciences at the University of Montana, and Director of the Center for Biomolecular Structure and Dynamics located there. The Board of Regents of the University of Texas System is the assignee of this patent application. I have authored numerous scientific papers in the field of protein structure and regulation. I have read and considered this patent application, the Decision dated Sept 19, 2007, and the cited references.

2. Prior descriptions (including Fesik, WO97/18471) of "SAR by NMR" wherein structure-activity-relationships are obtained by NMR, have invariably targeted "druggable" proteins, apo-proteins structurally characterized to have preformed ligand binding pockets, proteins such as FKBP, stromelysin, E2 DNA binding domain, Erm methyltransferase, SH2 domains, etc.

3. In contrast, the recited PAS domains are determined to be absent any ligand binding pocket, and such proteins have not been, and would not have been screened for ligand binding by NMR because based on their structure. Further, these domains do not require protein chaperones or other cellular components to adopt a stable fold, nor do they have known ligands. Finally, PAS domains are involved in protein/protein interactions (PPIs), making them members of a class of targets that are widely considered "undruggable": see; e.g. Whitty et al. Nature Chemical Biology 2, 112-118 (2006), p.112, first para.

4. In view of this prior knowledge and experience, one skilled in the art would not have determined that a candidate target protein in fact has no NMR apparent ligand binding site, and then turned around and initiated an NMR-based screen of that very target for ligand binding.

5. As explained in our Specification some members of the PAS family are known to contain small molecule cofactors within their cores, and these cofactors are reportedly required for proper folding and functioning of the PAS domain within the context of the holo-protein.

Specification, p.1, line 22 - p.2, line 1. However, for most PAS domains there is no evidence for such a cofactor. In fact, structurally characterized PAS domains without bound cofactors (Amezcua et al., 2002; Erbel et al., 2003; Morais Cabral et al., 1998) show tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site. Specification, p.2, lines 2-5.

6. Since the prior work provided no evidence of cofactors for most PAS domains, and taught that those limited PAS domains having cofactors required them for proper folding, and taught that PAS domains without cofactors had tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site, one skilled in the art would not have suspected that such PAS domains (without known cofactors and having tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site) would be rational candidates to screen for core ligand binding; in fact, the prior art teaches squarely away from such use.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.

Date: November 19, 2007

Professor Stephen R. Sprang

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Serial No. 10/677,733	Customer No.: 23379
Applicant: Gardner et al.	Confirmation No. 4887
Filed: Oct 01, 2003	Group Art Unit: 1656
Docket No. UTSD:1510	Examiner: Nashed, Nashaat T.

Title: NMR Detection of Foreign PAS Domain Ligands

DECLARATION UNDER 37CFR1.132

I, Professor Kevin H. Gardner, declare and state as follows:

1. I am an Associate Professor of Biochemistry and Pharmacology at the University of Texas Southwestern Medical Center in Dallas, where I also serve as the Chairman of the Molecular Biophysics Graduate Program. The Board of Regents of the University of Texas System is the assignee of this patent application. I have authored numerous scientific papers in the field of NMR analyses of protein structure, function and regulation. I am a coinventor on this patent application, and have read and considered the Final Action dated Dec 11, 2007, and Declaration of Professor Stephen Sprang dated Nov 19, 2007.

2. As someone skilled in the general area of biophysics, with over twenty years of expertise, predominantly in the area of NMR spectroscopy, I concur with the statements and opinions of Professor Sprang as provided in his Declaration dated Nov 19, 2007.

3. Furthermore, based on my expertise and carefully consideration, it is my conclusion that the commentaries about protein analysis put forth in the Final Action are not reflective of ordinary skill and understanding in the art. Its conclusions are unwarranted and erroneous, and its analysis contains multiple and fundamental overstatements and technical inaccuracies.

4. As detailed below, the Action fundamentally relies on two suppositions, both of which are technically incorrect: (a) that it is obvious to screen PAS domains because they all contain ligand binding sites; and (b) that solution NMR is an obvious choice of screening method to identify protein-binding ligands for any target.

5. "The ordinary skill in the art would have known of the presence of the ligand-binding cavity because the protein has an activity in solution." Action, p. 3.

This statement is both misleading and inaccurate. Indeed, most known PAS domains are not

naturally regulated by small molecule ligands or cofactors, and thus their activities in solution do not involve the binding of any such compounds. Examples include HERG (e.g. Cabral 1998), ARNT (e.g. Reisz-Proszasz et al., Mol Cell Biol 14(1994): 6075), and many others. The art teaches that these domains function as constitutive protein/protein interaction domains in their current settings, independent of natural small molecule regulation. Our innovation has been to establish a general way to test if these proteins might still be able to bind foreign core ligands despite their native disposition.

6. "[Whitty et al., 1997] takes the view that developing modulator protein activity involving protein/protein interaction is difficult, but not impossible. … [and Tiliey et al. 1997 provide] expectation of success to one of ordinary skill in the art that modulators of protein/protein interaction are possible to identify." Action, p. 4.

The accepted view in the field is that developing protein/protein inhibitors is difficult, but not always impossible, as shown by Tiliey 1997. But we are targeting internal binding sites that will have allosteric control of protein binding/activity, not the commonly-used route of directly binding the external protein-binding surface. Core targeting raises several additional challenges which made it a non-obvious route for an ordinarily skilled worker in the field:

- without an a priori formed cavity there would be an overwhelming expectation that our targeted core ligand binding sites would not even exist;
- it would be uncertain and unpredictable whether ligand binding to interior sites would be able to provoke an allosteric change that affects protein function, as opposed to the more straightforward options provided for exterior sites (e.g. direct occlusion of an external site is guaranteed to block function).
- compounds that target internal sites will likely have slow rates of binding ("on rates"), with correspondingly lower affinities than compounds which target the exterior surfaces.

With these challenges unique to our proposed core target sites, coupled with the difficult nature of finding protein/protein interaction inhibitors in general (supra), our application of detecting internal ligands would not have been, and was not obvious.

7. "The NMR method taught by Feisk [sic] is one of the most sensitive methods that detect the interactions between a protein and a small molecule … [A skilled person would] be motivated to use the most sensitive method of detecting the interaction, i.e., NMR." Action, bridging p. 2-3.

Persons with expertise in biophysical measurements of protein/small molecule interactions know that NMR spectroscopy is actually one of the least sensitive methods to look for such interactions. This is fundamentally rooted in the small energy separation between the ground and excited states that are probed by NMR spectroscopy – a small separation means small population differences, translating into poor sensitivity.

Despite recent advances in NMR magnet and probe technology, NMR still has a serious sensitivity disadvantage compared to alternatives. Practically, this means that NMR-based

screens looking for ligand-induced changes in protein signals generally require orders of magnitude more protein than competing technologies:

NMR:
typical sample: >100μM concentration, >300μL volume
= >3mg of 10kDa protein per sample
96-well thermal shift assays:
typical sample: 10μM protein concentration, 50μL volume
= 0.05mg of 10kDa protein per sample
384-well plate-based assay (e.g. PerkinElmer - AlphaScreen)
typical sample: 100nM concentration, 30μL volume
= 0.0003mg of 10kDa protein per sample

Furthermore, our protein-detected NMR assays require isotopically-labeled protein (usually ^{15}N or ^{13}C), and the only way to economically produce these proteins is by bacterial expression – *in vitro* or eukaryotic alternatives for these types of labeling are cost-prohibitive, even for large pharmaceutical companies. In addition, samples must be stable at relatively high concentration (>100μM) for extended periods of time (~ overnight) in 2-5% DMSO, should be < 30kDa, not prone to aggregation, etc.

Of course as an expert in the field, I appreciate that NMR has certain advantages. One of these, which I suspect the Examiner may be referring to in this context, is that NMR can uniquely detect low affinity binding (K_d > 1 mM, for example) as it is less prone to issues with background signals from high concentrations of free ligands compared to other methods. I also appreciate the fact that NMR methods give us resolved signals from a relatively large number of sites within a protein, allowing us to obtain some information about ligand binding site location(s) within a protein.

But even under the best of circumstances, NMR analysis is discouraged by its inherently poor sensitivity and the need to generate hundred milligrams of isotopically-labeled, stable protein. Here, it is flat-out contraindicated by the pre-confirmed absence of any NMR-apparent a priori formed ligand cavity.

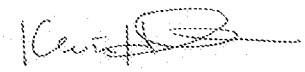
8. "Thus, one of ordinary skill in the art would not have been discouraged from using Feisk's [sic] method because the presence or absence of some NMR peaks indicating the absence or presence of a ligand-binding site." Action, p. 3.

The Examiner discusses the effect of dynamics on NMR spectra of proteins. He is right that these effects can lead to the disappearance of peaks in spectrum of a target, but these would discourage a practitioner in the field from proceeding with further studies of a protein target. The lack of peaks in this way is highly correlated with difficulty in further analysis and screens. One skilled in the art would not proceed with screening a sample using a certain method when that method does not provide the data needed to establish if ligands are binding or not.

9. "The major advantage of the NMR method over any other screening method is that it observes the binding of the small molecule directly to the target protein in its native environment, i.e. in aqueous solution. … There is no reason to believe that the most abundant conformation in solution which is observed by NMR is the most relevant conformation for binding a small molecule or a large target molecule." Action, p. 3

These sentences are inconsistent. The first argues that we should be using NMR methods for ligand screening so that we can work in a "native environment"; the second then argues that there is no reason to expect that the "most abundant" form of the protein under these conditions should be competent to bind ligand. This defies logic – why would I use a screening method that would inherently sacrifice sensitivity by having only a small fraction of the protein in a ligand-binding conformation? Either the NMR experiment is being performed under native conditions – in which case the dominant structure will tell us something that is worth screening – or the NMR experiment is not being performed under these conditions and its use is contraindicated. Here, NMR-based analysis of core ligand binding is flat-out contraindicated by the pre-confirmed absence of any NMR-apparent a priori formed ligand cavity.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application and any patent issuing therefrom.



Date: Apr 10, 2008

Professor Kevin H. Gardner

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Crystal Structure and Functional Analysis of the HERG Potassium Channel N Terminus: A Eukaryotic PAS Domain

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Summary

The HERG voltage-dependent K^+ channel plays a role in cardiac electrical excitability, and when defective, it underlies one form of the long QT syndrome. We have determined the crystal structure of the HERG K^+ channel N-terminal domain and studied its role as a modifier of gating using electrophysiological methods. The domain is similar in structure to a bacterial light sensor photoactive yellow protein and provides the first three-dimensional model of a eukaryotic PAS domain. Scanning mutagenesis of the domain surface has allowed the identification of a hydrophobic "hot spot" forming a putative interface with the body of the K^+ channel to which it tightly binds. The presence of the domain attached to the channel slows the rate of deactivation. Given the roles of PAS domains in biology, we propose that the HERG N-terminal domain has a regulatory function.

Introduction

HERG (human *eag*-related gene) is a member of the *eag* (*ether-a-go-go*) K^+ channel family (Warmke et al., 1991; Warmke and Ganetzky, 1994). These channels, found in human heart and nervous system, underlie one form of the long QT syndrome, LQT2 (Curran et al., 1995), a genetic condition causing familial cardiac arrhythmia and sudden death. In common with other voltage-dependent K^+ channels, HERG has a subunit topology of six membrane-spanning stretches (Figure 1A). Four of these subunits form a tetramer with a central ion conduction pore. Voltage-dependent gating, or opening and closing of the pore, is conferred by the S4 "voltage sensor" (the arginine-rich fourth membrane-spanning stretch) present in all members of the voltage-dependent cation channel family (Sigworth, 1994).

HERG exhibits two distinct and physiologically significant gating characteristics: rapid inactivation and slow deactivation (Trudeau et al., 1995; Smith et al., 1996; Spector et al., 1996). The presence of rapid inactivation means that when the channel is opened with cell membrane depolarization it very quickly enters a nonconducting (inactivated) state, passing very little current in the outward direction (Figure 1B). When the membrane is returned to its normal resting potential near −80 mV, the channel apparently retraces its conformational steps and passes through the open state on the way back to its closed configuration. The return to the closed state, referred to as the process of deactivation, is very slow in the HERG channel, and consequently a large inward "tail" K^+ current is observed during a voltage clamp experiment (Figure 1B). This slow rate of HERG channel deactivation plays an important role in cardiac electrical excitability by governing the length of the action potential (Sanguinetti et al., 1995).

Deletion of the HERG cytoplasmic amino terminus has been shown to profoundly affect the rate of deactivation (Schönherr and Heinemann, 1996; Spector et al., 1996; Terlau et al., 1997). This finding is very interesting because the amino terminus of HERG contains a sequence of about 135 amino acids, which is not only highly conserved, but is sufficiently unique to be considered a defining feature of the *eag* K^+ channel family (Warmke and Ganetzky, 1994). What structural unit is encoded by these amino acids, and why have they been conserved? To address these questions, we have solved the structure of the eag N-terminal domain (eag domain) by X-ray crystallography and have begun to characterize its interaction with the K^+ channel using site-directed mutagenesis and electrophysiology. The eag domain controls deactivation by tightly associating with the body of the K^+ channel, presumably through a hydrophobic patch on its surface. Its three- dimensional structure defines the first eukaryotic member of the PAS domain family. PAS (acronym for the gene products of *Per*, *Arnt*, and *Sim*) (Reppert, 1998; Sassone-Corsi, 1998) domains are found in proteins involved in the circadian rhythm, the cyclic patterns of hormone secretion, breeding, and locomotor activity in mammals and the oscillation of photosynthesis in plants. In prokaryotic cells, PAS domains regulate a variety of biochemical processes by serving as light and chemical sensors.

Results

Function of the eag Domain

The HERG potassium channel has a ~390 residue cytosolic N terminus, of which the first 135 form the eag domain. It was previously shown that removal of the eag domain results in active K^+ channels with altered gating properties (Schönherr and Heinemann, 1996; Spector et al., 1996; Terlau et al., 1997). Electrophysiological recordings from *Xenopus* oocytes injected with RNA encoding wild-type or eag domain–deleted HERG (truncated HERG) channels are shown in Figures 2A and 2B. The records focus on the tail currents where the deactivation process (transition back to the closed state) is observed. The deactivation rate is increased markedly

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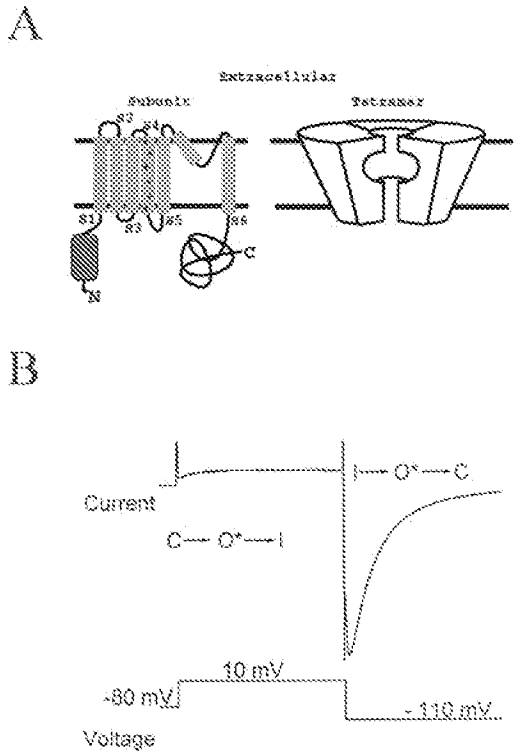

Figure 1. Membrane-Spanning Topology and Current Recorded from HERG K^+ Channels

(A) The HERG channel monomer has six transmembrane segments (blue) labeled S1 to S6; S4 is the voltage sensor, and it contains six positively charged amino acids as indicated by the + symbols. The channel has a large C terminus and the characteristic N-terminal eag domain (red), both cytosolic; the pore region is situated between S5 and S6. The functional channel is a tetramer with a central ion conduction pathway.

(B) Upon depolarization of the cell membrane to 10 mV from a holding voltage of −80 mV, the channels activate, passing from the closed state C to the open state O* (the * symbol indicates a conducting state) and ionic current is observed in the outward direction. Only a small amount of current is observed because the channels quickly enter the I (inactivated) state, which does not conduct ions. When the membrane is repolarized to −110 mV, the channels pass from the I state to the open state O* and a large inward "tail" current is recorded. This current slowly decays with an increasing number of channels deactivating or returning to the closed state C.

through removal of the eag domain. A double exponential function was fitted to the decay phase of the current traces, and the time constant for the faster, dominant component was graphed as a function of membrane voltage (Figure 2C). The truncated channel exhibits a faster rate of deactivation over voltages studied.

We next asked whether it was possible to reconstitute the wild-type function by application of eag domain produced in *E. coli* (Li et al., 1997) to the cytoplasmic surface of truncated HERG channels. The experiment was carried out in two ways. First, purified domain (corresponding to amino acids 1 to 135 of HERG) was applied at micromolar concentrations to an excised membrane patch containing many truncated HERG K^+ channels. The domain had no effect on channel gating in this acute exposure experiment (not shown). However, when the

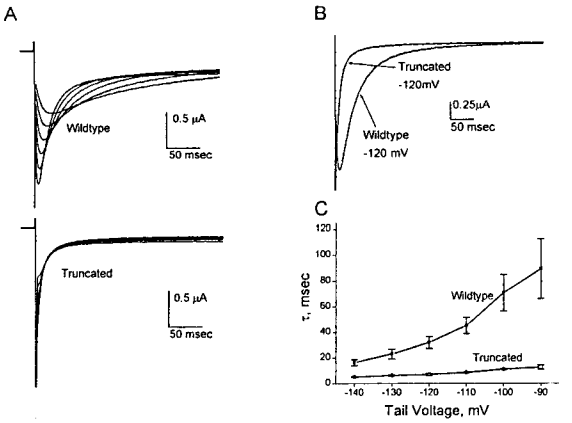

Figure 2. Deactivation of Wild-Type and eag Domain–Deleted (Truncated HERG) Channels

(A) After depolarization to 20 mV (not shown), tail currents for wild-type and truncated HERG channels were elicited by repolarizing the membrane to voltages between −140 mV and −90 mV in 10 mV increments.

(B) Currents recorded as in (A) at −120 mV, scaled to have nearly equal amplitudes, are superimposed.

(C) Plot of deactivation time constant as a function of the repolarization voltage. The time constant is that of the dominant fraction of a double exponential fit to the decay phase of the tail current. Error bars are standard error of the mean ($n = 3 - 5$).

eag domain protein was instead injected into oocytes expressing truncated HERG, the deactivation kinetics slowly converted to be wild-type-like (Figures 3A and 3B). Within 3 hr, the time constant for deactivation at −110 mV became two times slower, and further slowing progressed over 24 hr. Excision of membrane patches containing "wild-type-reconstituted" HERG showed no speeding of deactivation over a 3 min period (not shown); a rapid change in the deactivation rate upon excision would have been expected if the domain were loosely attached and exerted its effect by rapidly associating and dissociating with the channel. We therefore conclude that the eag domain must be bound tightly to the body of the channel.

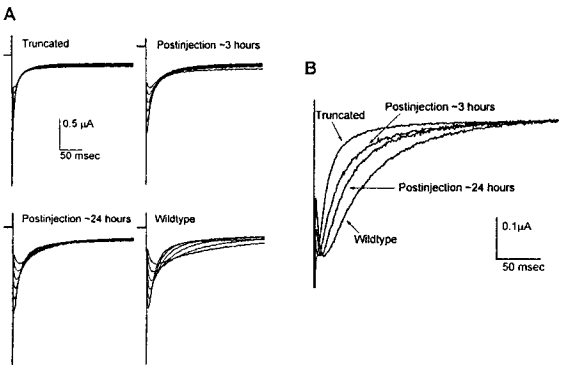

Figure 3. The eag Domain Confers Wild-Type Gating Properties

(A) Tail currents were recorded from oocytes expressing truncated HERG channels 3 and 24 hr after protein injection of eag domain protein. Currents from wild-type and truncated HERG are shown for comparison. The voltage protocol used is the same as in Figure 2A.

(B) Scaled currents recorded at −110 mV are superimposed.

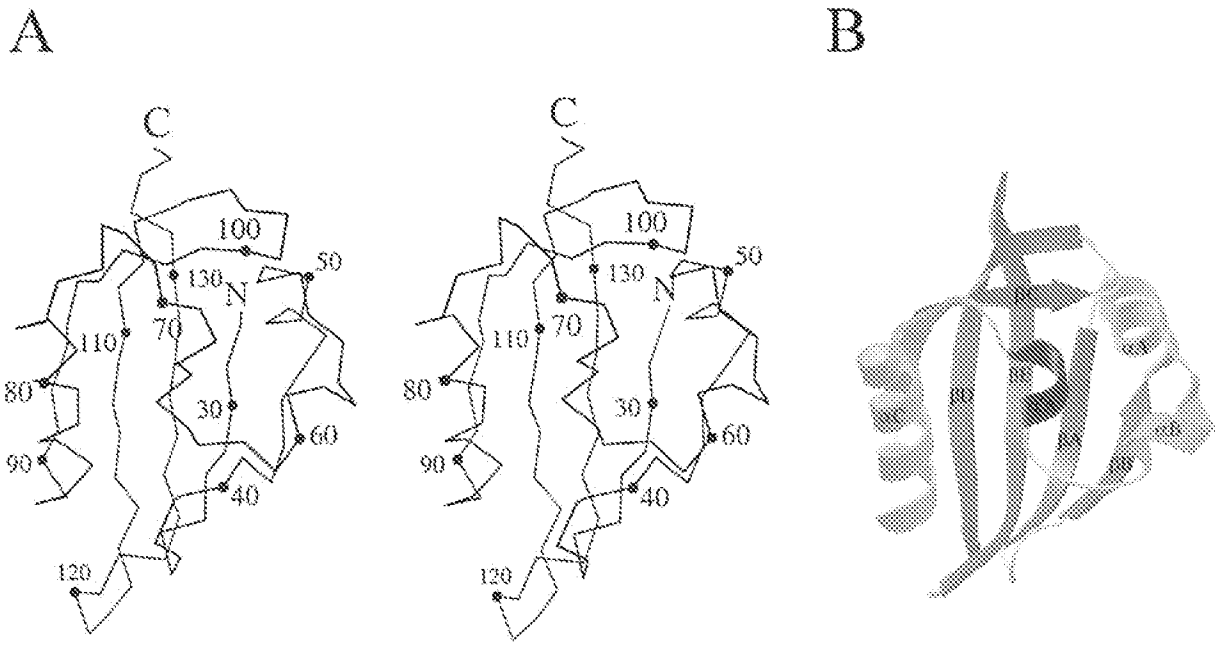

Figure 4. Stereo and Ribbon Diagrams of the eag Domain Three-Dimensional Model

(A) Stereo Cα plot with every tenth residue numbered and the N and C termini indicated.

(B) Ribbon diagram with secondary structure elements labeled. These pictures were drawn with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Bacon and Anderson, 1988).

One possible explanation for the effect of the eag domain on the deactivation kinetics is that it could be catalytic, modifying the channel or an effector of the channel already present in the cytoplasm. This indirect mechanism was tested by injecting eag domain into oocytes expressing a full-length channel containing a point mutation (F29A) within its own eag domain. This mutant channel deactivates at a rate that is intermediate between wild-type and truncated HERG, presumably because the F29A mutation renders the domain partially defective (see below). Injected eag domain did not slow deactivation. In other words, it did not compensate for the F29A mutant domain. The most likely explanation is that the mutated domain prevents attachment of the wild-type domain to the channel. This result reinforces the conclusion that the injected eag domain exerts its effect on gating by attaching tightly to the body of the HERG K^+ channel.

Crystal Structure

The structure of the eag domain was determined by a MAD (multiwavelength anomalous diffraction) experiment with selenomethionine substituted protein crystals at the CHESS F2 beamline. A three-dimensional model consisting of amino acids 26 to 135 and 27 water molecules was refined (Table 1) using data to 2.6 Å resolution with a free R value of 28.6% and a conventional R value of 25.2%.

The eag domain is an α + β protein with a five-stranded antiparallel β sheet (βA to βE) packed against a long ordered "vine" composed of coil and a single turn of 3_{10} helix (α′A) (Figures 4A and 4B). The sheet is decorated on two sides by α helices (αA to αC). The structure has its N and C termini positioned side by side forming the two central strands of the β sheet. Mass spectrometry confirmed that the crystals contain all 137 amino acids (the eag domain's 135 residues plus 2 residues from the fusion protein), indicating that the first 25 amino acids are disordered in the crystal. Additional (unmodelled) electron density is observed in both native and difference Fourier maps that might represent partially ordered segments of the missing N terminus. Interestingly, the disordered 25 N-terminal residues are among the most conserved in the eag domain (Warmke and Ganetzky, 1994). This conservation, together with the functional importance of the first 25 amino acids (see below) imply that this region may well be ordered in the context of the ion channel.

Mutational Analysis

In order to determine which eag domain residues establish the putative interface with the remainder of the HERG K^+ channel, we produced 15 point mutations spread over the domain surface and measured their effect on function (Figures 5A and 5B). All point mutations were to alanine except for Arg-73, which was mutated to cysteine. The role of the disordered (within the crystal) N terminus was studied by producing three deletion mutants: deletion of residues 2 to 26 (Δ2–26), 2 to 23 (Δ2–23), and 2 to 9 (Δ2–9). The effect of mutations was assessed by measuring the rate of deactivation as described above. The "speeding factor," defined as the ratio of time constants for the wild-type and mutant channels measured at −120 mV, is shown (Figure 5B). The mutants fall into three groups depending on the magnitude of effect. The largest group includes all mutations that have a speeding factor close to 1 (little or no

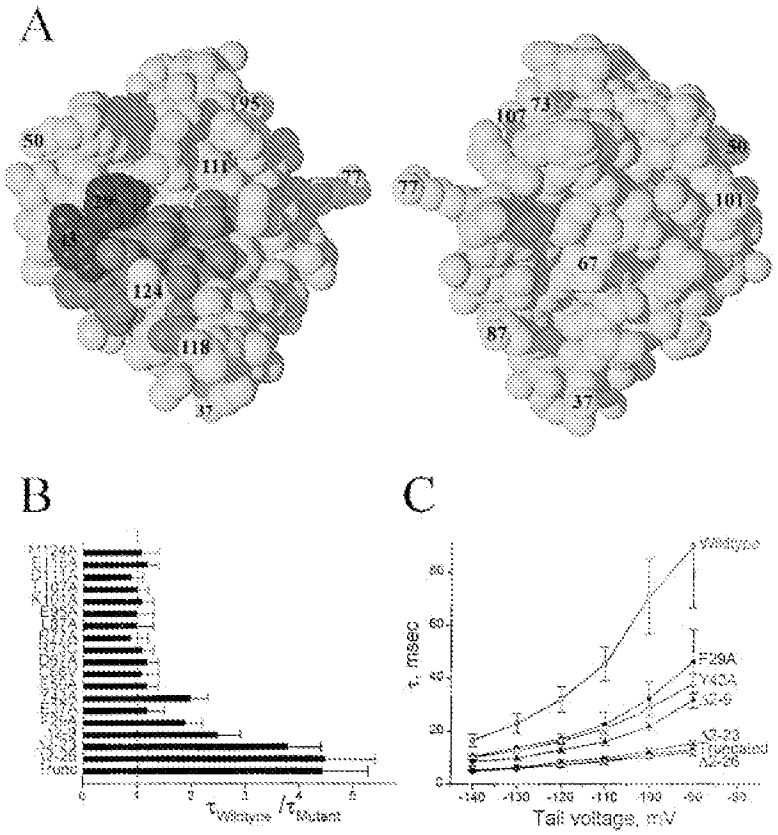

Figure 5. Mutational Analysis of the eag Domain

(A) Two views of a CPK model of the eag domain. The molecule is shown in the same orientation as in Figure 4 (right) and rotated by 180 degrees about a vertical axis (left). Mutated residues either affected (red; Phe-29 and Tyr-43) or did not affect (yellow) the deactivation rate. A hydrophobic patch is shown (green) and also includes Met-124, Phe-29, and Tyr-43. Picture drawn with BALLS (Merritt and Bacon, 1997).

(B) Bar plot of speeding factor (wild type-to-mutant time constant ratio) for deactivation at −120 mV. The dashed line marks a ratio of 1. Speeding factor for truncated HERG channels (Trunc) is also shown.

(C) Plot of deactivation time constant as a function of repolarization voltage for mutations that showed altered kinetics; data for wild-type and truncated HERG channels shown for comparison. Error bars are standard error of the mean ($n = 3 - 5$).

effect). A second group with a speeding factor close to 4 includes mutants Δ2–26 and Δ2–23, and a third with a factor around 2includes F29A, Y43A, and Δ2–9. Graphing the time constants over the full voltage range for the second and third groups (Figure 5C) shows that Δ2–26 and Δ2–23 are indistinguishable from truncated HERG, while the Δ2–9 mutant channel is intermediate between wild-type and truncated HERG, as are mutants F29A and Y43A.

The two point mutations affecting deactivation (F29A and Y43A) are clustered on the domain surface (Figure 5A). Moreover, these functionally important amino acids are located within a hydrophobic patch having a solvent accessible area of 530 Å^2 made up of residues Ile-31, Ile-42, Met-60, Val-113, Val-115, Ile-123, Met-124, and Ile-126, in addition to Phe-29 and Tyr-43. The functional importance of this patch is intriguing. In the crystal, the hydrophobic environment of the patch is maintained through packing of hydrophobic surfaces from two adjacent domains, resulting in a total buried surface area of 1190 Å^2 (both sides of the interface). However, gel filtration and analytical ultracentrifugation show the eag domain to be a monomer (not shown). Given that the domain appears to be tightly adherent to the body of the K^+ channel, the hydrophobic patch may provide the interface through which it binds.

The functional importance of the very N terminus of the eag domain is interesting particularly in light of previous results of Terlau and colleagues (Terlau et al., 1997). They showed in a rat eag K^+ channel that deletion of amino acids 7 to 12 altered the rate of deactivation and that the effect could be counteracted by a second mutation within the S4-S5 linker. The S4-S5 linker, that is, the connector between the fourth and fifth membrane-spanning segments, is a gating-sensitive region of voltage-dependent K^+ channels. Indeed, the S4 contains the basic amino acids underlying the voltage-dependent gate. We therefore speculate that when the eag domain is bound to the channel, the N-terminal amino acids are in a position to interact with residues in the S4-S5 linker. If this is the case, we might expect the N-terminal amino acids to become disordered when the eag domain is removed from the context of the channel.

The HERG N Terminus Is a Eukaryotic PAS Domain

The eag domain bares no structural resemblance to the tetramerization domain of the kV-type voltage-dependent K^+ channels (Kreusch et al., 1998), and in contrast to the tetramerization domain, the eag domain does not appear to self-oligomerize. The program DALI (Holm and Sander, 1994) was used to search the database for proteins with a three-dimensional structure similar to that of the eag domain. Photoactive yellow protein (PYP), a bacterial light-sensing protein, had a score three times higher than the next candidate, profilin. By aligning the β sheet of the two models (Figure 6A), it is clear that the eag domain and PYP have highly similar three-dimensional structures; the major difference between the two proteins occurs in the way the αC helix and associated "vine" pack against the β sheet. The rms deviation between the two structures for main chain atoms of residues marked by blue boxes in Figure 6B

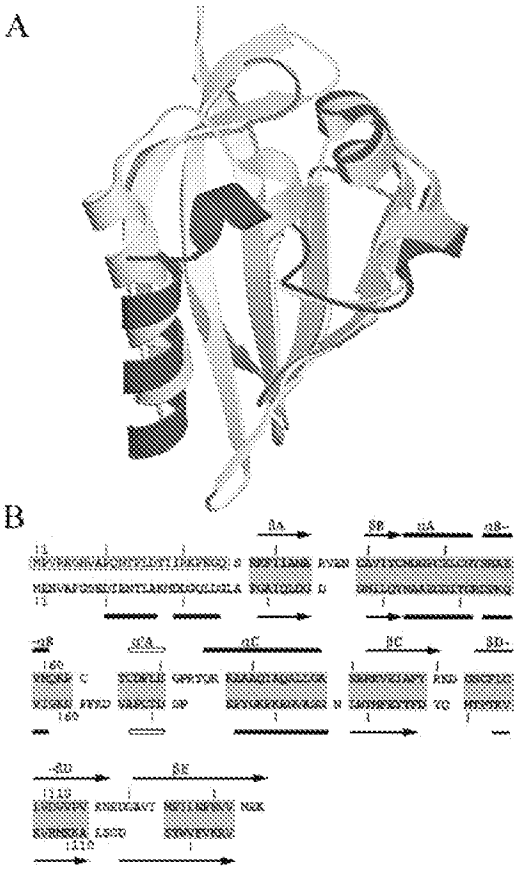

Figure 6. Structural Comparison of the eag Domain and Photoactive Yellow Protein (PYP)

(A) Ribbon diagram of superimposed eag domain (yellow) and PYP (blue) in same view as Figure 4.

(B) Sequence alignment of eag domain (top) and PYP according to the superimposed structures. The blue boxes mark stretches of sequence where aligned residues occupy the same position in secondary structure elements that are common to both structures. Secondary structural elements are marked above and below the corresponding sequence: α helices as arrows, β strands as filled rectangles, 3_{10} helix as open rectangle. The structural elements of the eag domain structure are labeled. The open red box marks residues that are disordered in the eag domain structure. Residue numbering is shown above and below the respective sequences.

is 2.5 Å; exclusion of residues corresponding to α′A and αC drops this value to 1.4 Å. A structure-based sequence alignment of the eag domain and PYP shows that there is no significant sequence conservation (Figure 6B); the relatedness of these proteins is apparent only through comparison of their three-dimensional structures.

PYP belongs to the PAS domain family and until now was the only member with a known structure. These proteins are present in prokaryotes where they serve as sensory domains and in eukaryotes where they participate in the biochemical pathways underlying the circadian rhythm, among other functions. Due to extreme sequence diversity, only recently have the PAS domains been proposed as a broad protein family (Ponting and Aravind, 1997; Zhulin et al., 1997; Pellequer et al., 1998).

Table 1. Data and Refinement Statistics

Data and Phasing Quality			
Data Set (13.0–2.6/2.7–2.6 Å)	L1 (12.660 keV)	L2 (12.665 keV)	L3 (12.740 keV)
Completeness (%)	95.8/87.9	95.9/87.9	95.9/88.1
R_{merge}[a] (%)	9.2/35.4	10.5/40.3	10.5/40.6
I/σI	34/5	32/4	33/4
Redundancy[b]	7/2.5	7/2.5	7/2.5
R_{cullis}[c] (anomalous)	0.83	0.51	0.74
R_{cullis}[d] (dispersive)	—	0.93	0.77
Overall FOM[e]	0.50		
Refinement Against L1 Data Set			
Resolution	13.0–2.6 Å		
$R_{crystallographic}$[f]	25.2%		
R_{free}[f]	28.6%		
Number of atoms			
Protein	817		
Waters	27		
No. of reflections with $F/\sigma F \geq 2$	3428		
Rmsd[g] bond angles	0.833°		
Rmsd[g] bond lengths	0.004 Å		
Rmsd[g] B factor for bonded atoms	1.088 Å^2		
Mean B factor for all atoms	33.2 Å^2		

I is the average intensity, Ij is the observed intensity, Dph is the observed anomalous difference, Dphc is the calculated anomalous difference, Fph is the observed amplitude for the derivative, Fp is the observed amplitude for the native, Fhc is the calculated amplitude for the heavy atom, Fpc is the calculated native amplitude.

[a] $R_{merge} = \Sigma\Sigma|I - Ij|/\Sigma Ij$.

[b] Redundancy was calculated for separate Bijvoets pairs.

[c] R_{cullis} (anomalous) = $\Sigma|Dph - Dphc|/\Sigma|Dph|$ for acentric reflections.

[d] R_{cullis} (dispersive) = $\Sigma|Fph - Fp + Fhc|/\Sigma|Fph - Fp|$ for centric reflections.

[e] FOM figure of merit.

[f] $R_{crystallographic} = \Sigma|Fp - Fpc|/\Sigma|Fp|$; R_{free}, the same, as $R_{crystallographic}$ but calculated on 14% of data excluded from refinement.

[g] Rmsd, root-mean-square deviation.

Through this study, we have defined a eukaryotic PAS domain structurally.

Discussion

In prokaryotic cells, PAS domains appear to have a sensory function. For example, PYP is a photoreceptor involved in the negative phototaxis response to blue light in certain bacteria (Borgstahl et al., 1995; Genick et al., 1997). It has a chromophore (4-hydroxycinnamyl) covalently bound to a cysteine in the "vine" and buried in the core of the protein; upon absorption of a photon, the chromophore isomerizes, inducing a conformational change on the protein surface. This change is thought to initiate a signal transduction process that affects the motility system of the bacterium. An *E. coli* membrane protein called aerotaxis provides a second example (Bibikov et al., 1997; Rebbapragada et al., 1997). The cytoplasmic N terminus of this protein contains a PAS domain implicated in sensing redox potential; the cytoplasmic C terminus communicates with the flagellar motor.

In eukaryotic cells, the best known PAS domains are

found in proteins involved in the circadian rhythm (Reppert, 1998; Sassone-Corsi, 1998). Monomeric forms of Per and Tim (*drosophila* clock gene products) are localized to the cytosol, whereas heterodimerization, mediated by the PAS domain present in Per, leads to translocation to the nucleus where they exert a negative feedback control on their own expression. Another eukaryotic PAS domain is present in the aryl hydrocarbon receptor (dioxin receptor). In this system, the PAS domain is thought to be involved in both ligand binding as well as in a protein–protein interaction (Hahn et al., 1997).

HERG and other members of the eag K^+ channel family contain a PAS domain on their cytoplasmic N terminus. Removal of this domain alters a physiologically important gating transition in HERG, and addition of isolated domain to the cytoplasm of cells expressing truncated HERG reconstitutes wild-type gating. Therefore, the domain finds the channel, attaches to it (presumably one domain per subunit through the hydrophobic patch on the domain surface), and confers its function. In summary, we conclude that the eag domain is a member of the PAS domain family and its role is to modify K^+ channel gating. Further, given the regulatory roles of PAS domains in other protein systems, we suspect that the eag domain will have a dynamic influence on the gating of the HERG K^+ channel through the binding of small molecule or protein effectors.

Experimental Procedures

Molecular Biology

The HERG channel was present in pSP64 vector between HindIII and BamHI sites. Mutations of the eag domain were generated by PCR mutagenesis across a HindIII/NcoI-flanked cassette; mutations were confirmed by automated sequencing. After DNA linerization with EcoRI, RNA was prepared by transcription with SP6 RNA polymerase.

Electrophysiology

Oocytes from *Xenopus laevis* were surgically removed and incubated for 1.5–2.5 hr in a solution containing: 1 mg/ml Collagenase type 2 (Worthington Biochemical Corporation), 82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH adjusted to 7.6 with NaOH. Defolliculated oocytes (stages 5 and 6) were injected with cRNA encoding wild-type or mutant HERG channel and incubated for 12–24 hr in a solution containing: 50 μg/ml gentamycin (GIBCO), 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH adjusted to 7.6 with NaOH.

Whole cell recordings were performed using a OC-725A two-electrode voltage clamp (Warner Instruments); the data were filtered at 1 kHz. Microelectrode resistances were 0.3–2.0 MΩ when filled with 3 M KCl. The oocytes were studied under continuous perfusion with a solution containing 58 mM NaCl, 40 mM KCl, 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM HEPES, pH adjusted to 7.6 with NaOH. All experiments were carried out at room temperature. Measurements were rejected if the tail current peak was larger than 3.0 μA and if the voltage electrode drifted by more than 10 mV at the end of the experiment. Patch clamp experiments were performed using an Axopatch 200A (Axon Instruments) amplifier; the data were filtered at 1 kHz. The oocytes were studied in a two-well bath containing 140 mM KCl, 0.5 mM EDTA, 10 mM HEPES, pH adjusted to 7.6 with KOH, with the reference electrode separated from the oocyte bath by a salt bridge. Reconstitution experiments were performed with eag domain protein expressed and purified as described below. Before injection, the protein was dialyzed overnight against a solution containing 140 mM KCl, 0.5 mM EDTA, 10 mM HEPES, pH adjusted to 7.6 with KOH. Oocytes expressing truncated HERG channel were injected with 100 nl of protein solution at a concentration of 70–90 μM.

Biochemistry

The eag domain (residues 1 to 135) was expressed as a glutathione-S-transferase (GST) fusion in *E.coli* BL21 (DE3) strain after overnight induction at 20°C. Cells were lysed in the presence of 0.1% Tween-20 (Pierce). The lysate was mixed with glutathione-Sepharose (Pharmacia), and the protein was eluted after thrombin digestion in a buffer containing 10 mM dithiothreitol (DTT) and 5 mM n-octyl-β-D-glucoside (Anatrace). Further purification was carried out by gel filtration in a Sephadex75 (Pharmacia) column equilibrated in a buffer containing 1 mM DTT without detergent. Final concentration was done in the presence of 10 mM DTT and 5 mM n-octyl-β-D-glucoside (analytical grade). Selenomethionine substituted protein was expressed and purified as described above with the following changes: B834(De3) *E. coli* strain, minimal media supplemented with essential amino acids except for methionine, which was substituted with selenomethionine. Mass spectrometry (MALDI-TOF) (Chait, 1994) was carried out to confirm that protein crystals were formed with full-length polypeptide and that full selenomethionine substitution occurred. Analytical centrifugation was performed in a Beckman Optima XL-A at three protein concentrations in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM n-octyl-β-D-glucoside, 1 mM DTT.

Crystallization and Crystallographic Methods

Crystals were grown at 20°C by sitting-drop vapor diffusion. Protein solution at ~10 mg/ml in 10 mM DTT, 5 mM n-octyl-β-D-glucoside, 150 mM NaCl, 50 mM Tris (pH 8.0) was mixed with an equal volume of reservoir solution: 0.8–1.0 M sodium and potassium tartrate, 100 mM HEPES (pH 7.0). The eag domain was crystallized in spacegroup $P6_522$, with cell dimensions $a = 56.1$ Å, $c = 135.5$ Å and one molecule in the asymmetric unit. The crystals were difficult to reproduce and suffered from nonisomorphism, making heavy-atom search a difficult task. All diffraction data for the MAD (multiwavelength anomalous diffraction) experiment were collected on a single selenomethionine-substituted protein crystal at Cornell High Energy Synchrotron Source (CHESS) at F2 station on a ADSC Quantum-4 CCD camera. Some native data were also collected at X12-C station at the National Synchrotron Light Source. Crystals were flash-frozen in freshly thawed liquid propane after being cryoprotected in crystallization solution containing increasing amounts of glycerol (5%–20%). After wavelength calibration and fluorescence scan of a wet-mounted crystal, six data sets were collected with inverted beam geometry at three energies (inflection point, 12.660 keV; peak, 12.665 keV; and remote, 12.740 keV). Data were integrated with DENZO, and scaling was performed in SCALEPACK (Otwinowski, 1993) in two steps. First, scale and B factors were calculated simultaneously for all data images. These factors were then used for internal scaling of each wavelength data set. Further processing was done with the CCP4 package (CCP4, 1994). SHELX (Sheldrick, 1990) was used to determine the position of the first selenium by direct methods and Patterson search. Initial phases were calculated in MLPHARE (CCP4, 1994), and two more selenium positions were confirmed on difference maps. Phases were calculated in MLPHARE using the inflection point data set as the native reference (Table 1). After solvent-flattening in DM (Cowtan, 1994), an electron density map of excellent quality was calculated that permitted the tracing of most of the model. Refinement (Table 1) consisted of rounds of model building in the program O (Jones et al., 1991) followed by bulk solvent correction, positional refinement (phase restraints were included in some of the cycles), and restrained B factor refinement with X-PLOR (Brunger, 1996). Electron density difference maps confirmed the presence of unaccounted density in the native map; this density might be attributed to the disordered N terminus. The final model includes residues 26 to 135 and 27 water molecules. The following side chains have been truncated: Ser-26 to Cα, Arg-35 to Cγ, Glu-37 to Cβ, Gln-75 to Cδ, Arg-76 to Cβ, Arg-77 to Cβ, Gln-84 to Cβ, Glu-90 to Cβ, Glu-118 to Cβ, Asp-119 to Cα, and Glu-130 to Cγ. According to PROCHECK (CCP4, 1994) 89.8% of the main chain torsional angles are in the most favored regions of the Ramachandran plot and none in the generously allowed or disallowed areas.

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Brookhaven Protein Data Bank ID Code

Coordinates of the refined HERG potassium channel PAS domain have been deposited in the Protein Data Bank (Brookhaven Laboratory) with ID code 1byw.

RELATED PROCEEDINGS APPENDIX

Decision in Appeal 2007-2956 in this application, decided Sep 19, 2007.

The opinion in support of the decision being entered today
is *not* binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

**BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES**

Ex parte KEVIN H. GARDNER, CARLOS A. AMEZCUA,
PAULUS J.A. ERBEL, and PAUL B. CARD

Appeal 2007-2956
Application 10/677,733
Technology Center 1600

Decided: September 19, 2007

Before TONI R. SCHEINER, DEMETRA J. MILLS, and RICHARD M. LEBOVITZ, *Administrative Patent Judges*.

LEBOVITZ, *Administrative Patent Judge*.

DECISION ON APPEAL

This is a decision on appeal from the final rejection of claims 1 and 2. We have jurisdiction under 35 U.S.C. § 6(b). We affirm.

STATEMENT OF THE CASE

"PAS (Per-ARNT-Sim) domains are protein interaction domains widely used for intra- and intermolecular associations. . . . Some members of the PAS family are known to contain small molecules within their cores, allowing them to sense stimuli and regulate diverse biological processes.

For example, heme binding by the PAS domains of FixL . . . allows bacteria to sense oxygen levels" (Spec. 1). "However, for most PAS domains there is no evidence for such a cofactor. In fact, structurally characterized PAS domains without bound cofactors (Amezcua et al., 2002; Erbel et al., 2003; Morais Cabral et al., 1998) show tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site" (Spec. 2). According to the Specification, "the invention provides methods of detecting binding of a PAS domain with a foreign core ligand of the PAS domain, wherein the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity" (Spec. 2).

Claims 1 and 2 are pending (App. Br. 1). Claim 1 is rejected under 35 U.S.C. § 103 as obvious over Fesik (WO 97/18471, May 27, 1997) in view of Edery (US 5,843,683, Dec. 1, 1998), Takahaski (US 6,291,429 B1, Sep. 18, 2001), or Berkenstam (US 6,436,654 B1, Aug. 20, 2002) (Answer 3). Claim 2 is objected to because it is dependent on rejected claim 1, but the Examiner states it would be allowed if rewritten in independent form (Answer 3). Claim 1 reads as follows:

> 1. A method of detecting binding of a PAS (Per-ARNT-Sim) domain with a foreign core ligand of the PAS domain, wherein the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity, the method comprising the steps of:
>
> detecting a first NMR spectrum of the PAS domain in the presence of a foreign ligand; and
>
> comparing the first NMR spectrum with a second NMR spectrum of the PAS domain in the absence of the ligand to infer the presence of the ligand specifically bound within the hydrophobic core of the PAS domain.

DISCUSSION

"[T]he Examiner bears the initial burden, on review of the prior art . . . , of presenting a prima facie case of unpatentability. If that burden is met, the burden of coming forward with evidence or argument shifts to the applicant." *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed, Cir. 1992). *See* also *Hyatt v. Dudas*, 492 F.3d 1365, 1369-70, 83 USPQ2d 1373, 1375-76 (Fed. Cir. 2007).

The Examiner finds:

1) Each of Edery, Takahaski, and Berkenstam describe PAS domain proteins with hydrophobic cores, satisfying the limitation of claim 1 of a PAS domain which is "predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity" (Answer 4-6).

2) Edery, Takahaski, and Berkenstam teach identifying compounds which modulate the activity of the PAS domain protein (Answer 4-5).

3) Fesik teaches a method of identifying compounds which bind to proteins using NMR spectra (Answer 3).

The Examiner contends that it would have been obvious to have used Fesik's NMR method to identify compounds which modulate the PAS domain proteins of Edery, Takahaski, and Berkenstam because Fesik teaches that its method is "amendable to automation for identification of modulator of protein activity" (Answer 5).

The Examiner's case for prima facie obviousness is built on the presumption that the proteins described in each of Edery, Takahaski, and Berkenstam satisfy the claimed limitation of a PAS domain which is "predetermined, prefolded in its native state, and comprises a hydrophobic

core that has no NMR-apparent a priori formed ligand cavity." When the Patent Office has reason to believe that a functional limitation asserted to be critical for establishing patentability is possessed by the prior art, "it possesses the authority to require the applicant to prove that the subject matter shown to be in the prior art does not possess the characteristic relied on." *In re Swinehart*, 439 F.2d 210, 212-13, 169 USPQ 226, 228-29 (CCPA 1971). *See* also *In re Best*, 562 F.2d 1252, 1254-55, 195 USPQ 430, 433-34 (CCPA 1977); *In re Spada*, 911 F.2d 705, 709, 15 USPQ2d 1655, 1658 (Fed. Cir. 1990). Thus, the issue in this appeal is whether there is a reasonable basis for believing that the prior PAS domain proteins meet the claimed limitation of "the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity."

In explaining the reason for the presumption that the prior art meets this claim limitation, the Examiner states:

> The linear amino acid sequence contains all the information required for proper folding of the protein to predetermined three-dimensional structure including any binding cavity required for its activity. Proteins are known to instantaneously fold during their biosynthesis. All folded proteins have hydrophobic core otherwise the protein would not fold.

(Answer 6.) Thus, the Examiner clearly states a reasonable basis for presuming that the PAS domain is in a "predetermined, prefolded in its native state, and comprises a hydrophobic core" as recited in claim 1. Appellants have not identified a defect in this reasoning, and we find none as it accurately reflects the knowledge of persons of ordinary skill in the art.

The claim further requires that the PAS domain "has no NMR-apparent a priori formed ligand cavity." We acknowledge that none of the

references cited for the teaching of PAS domain proteins describe the properties of the protein when imaged by NMR. With no explicit disclosure on whether the prior art PAS proteins possess an "NMR-apparent a priori formed ligand cavity," the first task is to determine whether there is any information that would lead persons of skill in the art to reasonably believe they do not as required by claim 1.

As pointed out by the Examiner, and not challenged by Appellants, the prior art PAS domains comprise a hydrophobic core. Persons of skill in the art would know that hydrophobic regions of a molecule would bond together, bringing the regions in close contact with each other.[1] In our opinion, this configuration would reasonably lead persons of skill in the art to infer that such regions do not have a ligand cavity in the native state, and that therefore, such cavity would not be detected by NMR, satisfying the limitation of claim 1.

In reaching this conclusion, we acknowledge that the Examiner erred in finding that "the PAS domains of the cited [prior] art must contain a binding cavity" (Answer 6; *see* Reply Br. 3, stating that the Examiner's assertion is "contrary" to the evidence of record). However, we do not find this misstatement fatal to the rejection. Nonetheless, because we have supplemented the rejection with reasoning of our own, we designate it as a new ground of rejection under 37 C.F.R. § 41.50(b).

In sum, we find that prima facie obviousness of the claimed subject matter has been established. First, the Examiner has provided a proper reason for combining the cited prior art. Secondly, a rationale has been

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[1] Darnell, *Molecular Cell Biology* 26 (2nd Edition, 1990).

provided to explain why it is reasonable for skilled persons in the art to believe that the prior art PAS domain proteins possesses the claimed limitation of "the PAS domain is predetermined, prefolded in its native state, and comprises a hydrophobic core that has no NMR-apparent a priori formed ligand cavity." Thus, there is sufficient evidence to shift the burden to Appellants to show that the claimed subject matter does not possesses the recited limitation.

Appellants contend that

> the prior work provided no evidence of cofactors for most PAS domains, and taught that those limited PAS domains having cofactors required them for proper folding, and taught that PAS domains without cofactors had tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site, one skilled in the art would not have suspected that such PAS domains (without known cofactors and having tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site) would be rational candidates to screen for core ligand binding; in fact, the art (supra) teaches squarely away from such use.

(App. Br. 5.)

We do not agree that "one skilled in the art would not have suspected that . . . PAS domains (without known cofactors and having tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site) would be rational candidates to screen for core ligand binding" (Appeal Br. 5). Takahaski suggests a method for identifying ligands for a PAS protein having a hydrophobic core (Answer 6). Edery also describes an assay method for identifying compounds that regulate a PAS domain protein's activity. Thus, despite the fact that these proteins have tightly packed cores with no pre-formed cavities – a fact that Appellants have not challenged – it was still suggested that these PAS domain proteins be

utilized for ligand screening (see, e.g., Takahaski, at col. 9, ll. 14-16; Edery, at col. 46-50).

Appellants state that a Declaration has been provided "documenting the fact that one skilled in the art would have considered the claimed invention nonobvious at the time it was made" (App. Br. 5). Paragraph No. 4 of the declaration (Declaration under § 1.132 by Dr. Stephen Sprang) repeats the same argument set forth in the Appeal Brief that we have already found to be unpersuasive.

Dr. Sprang states that he is "familiar" with the instant patent application, but he does not indicate his familiarity with the rejection at issue in this appeal nor the references cited in it. Moreover, he makes no mention of the references cited in the § 103 rejection, nor has he explained why the claimed invention is nonobvious over them. For this additional reason, we do not find the declaration sufficient to rebut the rejection.

The Specification refers to various prior art publications, including Morais Cabral (*Cell*, 95:649-655, 1998), for teaching "structurally characterized PAS domains without bound cofactors (. . . Morais Cabral et al., 1998) showing tightly packed cores with no pre-formed cavities that would suggest a cofactor or ligand binding site" (Spec. 2: 2-5). Morais Cabral, which Appellant admits satisfies the claim limitations for a PAS domain (App. Br. 5), compares the eag PAS domain of the HERG potassium channel to other PAS domain proteins known to comprise a ligand in their hydrophobic core (*see id.*, at 852, col. 2, describing the PYP photoreceptor which has a chromophore associated with its PAS domain). Morais Cabral conclude: "[g]iven the regulatory roles of PAS domains in other protein systems, we suspect that the eag domain will have a dynamic influence on

the gating of the HERG K^+ channels through the binding of small molecule or protein effectors" (*id.*, at 854, col. 2). Thus, despite having a tightly packed core with no pre-formed cavity, in view of its similarity to other PAS domain proteins, Morais Cabral suggested that small molecules might regulate eag domain activity as they do for other PAS domains.

In sum, we conclude that Appellants did not sustain their burden in rebutting the case of prima facie obviousness of claim 1. The rejection is affirmed.

TIME PERIOD

This decision contains a new ground of rejection pursuant to 37 C.F.R. § 41.50(b) (effective September 13, 2004, 69 Fed. Reg. 49960 (August 12, 2004), 1286 Off. Gaz. Pat. Office 21 (September 7, 2004)). 37 C.F.R. § 41.50(b) provides "[a] new ground of rejection pursuant to this paragraph shall not be considered final for judicial review."

37 C.F.R. § 41.50(b) also provides that the Appellant, WITHIN TWO MONTHS FROM THE DATE OF THE DECISION, must exercise one of the following two options with respect to the new ground of rejection to avoid termination of the appeal as to the rejected claims:

> (1) Reopen prosecution. Submit an appropriate amendment of the claims so rejected or new evidence relating to the claims so rejected, or both, and have the matter reconsidered by the Examiner, in which event the proceeding will be remanded to the Examiner. . . .
>
> (2) Request rehearing. Request that the proceeding be reheard under § 41.52 by the Board upon the same record. . . .

Should the Appellants elect to prosecute further before the Examiner pursuant to 37 C.F.R. § 41.50(b)(1), in order to preserve the right to seek

review under 35 U.S.C. §§ 141 or 145 with respect to the affirmed rejection, the effective date of the affirmance is deferred until conclusion of the prosecution before the Examiner unless, as a mere incident to the limited prosecution, the affirmed rejection is overcome.

If the Appellants elect prosecution before the Examiner and this does not result in allowance of the application, abandonment or a second appeal, this case should be returned to the Board of Patent Appeals and Interferences for final action on the affirmed rejection, including any timely request for rehearing thereof.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a).

AFFIRMED; § 41.50(b)

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